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(54) **Laminar bone support for cartilage growth**

Laminare Knochengrundlage für das Knorpelwachstum

Support d'os laminaire pour la croissance du cartilage

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- **BETTEX-GALLAND M. ET AL.: "Differentiation of L6 myoblastic cells into chondrocytes" EXPERIENTIA, vol. 43, no. 6, 1987, SWITZERLAND, pages 610-611, XP002057504**

EP 0 739 631 B1

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Description

[0001] The present invention relates to reconstituted cartilage tissue; to a method for producing reconstituted cartilage tissue by use of a biocompatible support material as a substrate for impregnation with living cells to enable cell growth and division; and to cartilage tissue reconstituted *in vitro* from isolated chondrocytes.

[0002] This invention is primarily directed to reconstituted articular cartilage, which is a specialized tissue found at the end of articulating bones. Cartilage, unlike other connective tissues, lacks blood vessels, nerves, lymphatics and basement membrane. It is responsible for the distribution of load resistance to compressive forces, and the smooth gliding that is part of joint function.

[0003] Cartilage is composed of chondrocytes which synthesize an abundant extracellular matrix, which is composed of water, collagens, proteoglycans and non-collagenous proteins and lipids. Collagen serves to trap proteoglycans and to provide tensile strength to the tissue.

[0004] Damage of cartilage produced by disease, such as arthritis, or trauma is a major cause of physical deformity and debilitation. In medicine today, the primary therapy for loss of cartilage is replacement with a prosthetic material, such as silicone for cosmetic repairs, or metal alloys for joint relinement. Placement of prostheses is commonly associated with significant loss of underlying tissue and bone without recovery of the full function allowed by the original cartilage, as well as the irritating presence of a foreign body. Other long term problems associated with a permanent foreign body can include infection, erosion and instability.

[0005] Very little has ever been actually used to replace the cartilage overlaying bone surfaces. To date, the growth of new cartilage from either transplantation of autologous or allogeneic cartilage has been largely unsuccessful. Microscopic islands of cartilage formation have recently been demonstrated histologically *in vivo* by implanting recombinant bone morphogenic protein, as reported by J. M. Wozney, et al., Science, 242, 1528-1534, (Dec. 16, 1988). Limited success has been achieved in making neocartilage using free autogenous grafts of perichondrial flaps, as described by J. Upton, Plastic and Reconstructive Surgery, 68(2), 166-174, (August 1981).

[0006] It is also known in the art to use cells harvested from an organism for implantation into the same or a different organism. Such techniques are shown in U.S. Pat. Nos. 4,299,819, 4,418,691, 4,458,678, 4,505,266, 4,553,272, 4,804,382, 4,883,487, 4,904,259, 4,960,423, 5,015,584, 5,035,708, and 5,041,138. In these references, the cells are seeded on a biocompatible support material and implanted into a desired site for tissue repair, with or without a step of cell cultivation *in vitro*, depending upon the type of tissue being replaced and the extent of tissue damage at the site need-

ing repair. The limitations of this technique are associated with the substrate material. Typically, bioabsorbable substrates are used for cell seeding. During cell cultivation *in vitro*, portions of these substrate materials are absorbed, leaving a support material for implantation which does not have the strength and resiliency to function as a prosthetic device at the site of tissue damage while additional tissue ingrowth and cell division takes place.

[0007] The provision of a suitable support material is a critical part of the *in vitro* cell cultivation technique. This is because most types of tissue will not grow or divide in suspension; a solid support is necessary for the cells to perform their normal functions. However, because the growing cells must receive nutrients from the nutrient media and eliminate cell waste products, all without a circulatory system which normally performs this function, the support upon which the cells are placed must allow for this exchange. Additionally, many cell cultures require a support coated with materials which form a part of the extracellular matrix, a collection of polysaccharides and proteins which exist outside the cell's membrane.

[0008] Cheung (*in vitro* Cell. Dev. Biol. 21:353, 1985) teaches a method of culturing canine chondrocytes on porous hydroxyapatite ceramic granules. The cells reportedly proliferated and secreted metachromatic extracellular matrix for up to 13 months. An agarose gel matrix has also been described as suitable for the *in vitro* culture of human chondrocytes (Delbruck et al., Conn. Tiss. Res. 15:155, 1986). Watt and Dudhia (Differentiation 38:140, 1988) disclose a composite gel of collagen and agarose for the culture of porcine chondrocytes. The composite gel prevented chondrocytes spreading. However, virtually no extracellular matrix was secreted in the low density culture composite gels.

[0009] Macklis et al. (*in vitro* Cell. Develop. Biol. 21: 180, 1985) teach a collagen surface for culturing peripheral nervous system cells, comprising collagen derivatized to polystyrene plastic culture dishes. Macklis et al. disclose that the derivatized coating process yielded enhanced collagen adhesion and increased long term survival of cultured nerve cells, compared to collagen coating produced by absorption techniques.

[0010] U.S. Patent No. 5,376,118, relates to the use of a support material fabricated from semiabsorbable, composite yarn as a support for cell growth *in vitro*. The support comprises a nonabsorbable, elastic core yarn and at least one absorbable, relatively inelastic sheath yarn imparting transverse strength to the composite yarn. The cells that can be grown on the support allegedly encompass endothelial cells, epidermal cells, muscle cells, bone cells, and cartilage cells.

[0011] U.S. Patent No. 5,326,357, relates to the use of a substrate for chondrocyte cell growth *in vitro*. The substrate that is employed is a synthetic substrate, Millicell®-CM, coated with attachment factors.

[0012] U.S. Patent No. 5,041,138, relates to a method

for making a cartilaginous structure by use of a biocompatible, biodegradable synthetic polymeric matrix for chondrocyte cell growth *in vitro*, and its use for replacing defective or missing cartilage.

[0013] To date, none of the aforementioned methods of cartilage growth and replacement have found wide acceptance. As can be seen from the descriptions, the methods that use chondrocyte cell growth *in vitro*, rely upon synthetic support media that are foreign to the body, resulting in problems associated with the introduction of foreign compositions into the body.

[0014] A need therefore exist for a method for growing chondrocytes *ex vivo*, by use of a supportive substrate that will find universal acceptance upon implantation for reconstructive use, while providing the characteristics necessary for cell growth *in vitro*.

[0015] The present invention provides a method for growing chondrocytes *ex vivo*, by use of a supportive substrate that is uniquely biocompatible, making the resulting structure with cartilage growth thereon especially useful for implantation for reconstructive use, while the structure provides the characteristics necessary for cell growth *in vitro*.

[0016] In accordance with the present invention, the supportive substrate that is used for chondrocyte growth *in vitro* is a continuous, flexible sheet of demineralized natural bone, having a thickness less than about 1.5 mm. As used herein below, all reference to use of a substrate is meant to refer to the aforementioned flexible sheet of demineralized bone.

[0017] Use of such a substrate provides a surface with physiological orientation of collagen fibers and physiological micro-anatomy and micro-porosity. This provides chondrocytes with a physiological receptor surface for attachment and allows for good exchange of nutrients and gases approximately equivalent to that which occurs *in vivo*.

[0018] The surface of the substrate, which is pliable and flexible, easily adopts to the surface of exposed subchondral bone, which can be microporated to stimulate outward migration of undifferentiated mesenchymal cells of bone marrow. Because the substrate is a membrane made from demineralized bone it is able to act as an osteoinductive agent. Thus, undifferentiated mesenchymal cells coming in contact with the substrate differentiate into cartilage and bone producing cells. Eventually the substrate, after implantation become replaced by new live bone, which unites with exposed subchondral bone and provides a physiological support for *in vitro* grown chondrocytes. Total replacement of the substrate in normal conditions occurs within six months after implantation.

[0019] Thus an advantage of the use of the present substrate over synthetic membranes is the physiological orientation of the resulting collagen fibers, and physiological micro-anatomy of the bone itself that is present in the substrate. This provides physiological receptor surface for attachment and proliferation of chondrocytes

in vitro. Physiological micro-porosity of human bone thus provides optimal exchange of nutrients and gases.

[0020] After the implantation, the substrate demonstrates osteoinductive phenomena and instead of being resorbed by ingrowing blood vessels, becomes replaced by new live bone within six-month period. Replacement of the substrate with new live bone provides chondrocytes with physiological attachment base through which physiological exchange of nutrients and gases occurs.

[0021] According to a first aspect, the present invention provides a method for producing a biological material comprising reconstituted cartilage tissue, wherein the method comprises isolating chondrocytes from cartilage tissue, contacting a substrate comprised of a continuous, flexible sheet of demineralised natural bone, having a thickness less than about 1.5mm, with said chondrocytes, and culturing the chondrocytes on the substrate in growth media to produce a biological material characterized by a continuous layer of cartilage tissue.

[0022] In another aspect, the invention provides a method for making a cartilaginous structure comprising providing a substrate comprised of a continuous, flexible sheet of demineralised natural bone, having a thickness less than about 1.5mm, in a nutrient environment and attaching cartilage cells to the substrate *in vitro* to form a cartilaginous structure suitable for implantation into a patient to replace defective or missing cartilage.

[0023] The chondrocytes used in these methods may be obtained by removing cartilage tissue, particularly articular cartilage, from the joint of an animal; and digesting said cartilage tissue to obtain isolated chondrocytes.

[0024] Subsequently, a monolayer of chondrocytes is formed on the substrate and the chondrocytes are cultured in growth media to produce a tissue having a biochemical composition and physiological organization substantially the same as articular cartilage tissue.

[0025] In a further aspect, the invention relates to a biological material comprising cartilage tissue and a substrate, the material being obtainable by reconstituting cartilage tissue on a substrate *in vitro* from isolated chondrocytes wherein the substrate is a continuous, flexible sheet of demineralised natural bone, having a thickness less than about 1.5mm.

[0026] Once again isolated chondrocytes can be obtained by digesting cartilage tissue. A monolayer of chondrocytes is then formed on the substrate and the chondrocytes are cultured in growth medium to produce a tissue having a biochemical composition and physiological organization substantially the same as normal mammalian cartilage tissue.

[0027] The reconstituted cartilage tissue structure of the present invention can be used to test pharmaceutical preparations for efficacy in the treatment of diseases of the joint and also as an Implant to replace or repair damaged or deficient cartilage.

[0028] Embodiments of the invention are described

below by way of example only, and with reference to the accompanying figures, of which:

[0029] Fig. 1 shows chondrocytes that have grown *in vitro* as multilayer structure closely adhering to the surface of the substrate.

[0030] Fig. 2 shows chondrocyte growth that follows the contour of the substrate even in perpendicular directions. Growth of chondrocytes extends into Haversian channels which constitute a natural porosity of the substrate. This results in very tenacious attachment between new cartilage and the substrate.

[0031] As indicated above the substrate used in accordance with the present invention comprises a continuous, flexible sheet of demineralized natural bone, having a thickness less than about 1.5 mm. Such a structure is taught and can be obtained in accordance with U.S. Patent No. 5,306,304.

[0032] There are several kinds of cartilage. Hyaline cartilage is a bluish-white, glassy translucent cartilage having a homogeneous matrix containing collagenous fibers which is found in articular cartilage, in costal cartilages, in septum of the nose, in larynx and trachea. Articular cartilage is hyaline cartilage covering the articular surfaces of bones. Costal cartilage connects the true ribs and the sternum. Fibrous cartilage contains collagen fibers. Yellow cartilage is a network of elastic fibers holding cartilage cells which is primarily found in the epiglottis, the external ear, and the auditory tube. As described below, cartilage implants can be formed of one or more types of cartilage, depending primarily on the location of the implant and the type of cartilage cells seeded onto the substrate.

[0033] In one embodiment the substrate is placed in culture media containing chondrocytes, where the chondrocytes attach to the fibers in multiple layers. Usually, the chondrocytes retain their normal rounded configuration, which appears to be useful for the chondrocytes to maintain their normal function and secrete a cartilage matrix and other bioactive molecules such as angiogenesis inhibiting factor.

[0034] The composite structure of substrate and cells can be transplanted into animals without disrupting the complex of attached chondrocytes. Transplantation of this composite containing a high density of normally functioning chondrocytes with a large surface area into an animal allows the cells to obtain adequate nutrition by diffusion and successful engraftment of functioning chondrocytes with cartilage formation even in the initial absence of vascularization.

[0035] Chondrocytes are initially isolated and cultured using techniques known to those skilled in the art of tissue culture. In contrast to some types of cells, chondrocytes can be seeded directly onto the substrate and implanted without first proliferating the cells *in vitro*. If insufficient cell numbers are available for implantation, cells are first cultured *in vitro* on the matrix. Once the cells have begun to grow and cover the matrix, they can be implanted in a patient at a site appropriate for attach-

ment, growth and function.

[0036] Because the substrate will eventually be reabsorbed, angiogenic and other bioactive compounds can be incorporated directly into the substrate so that they are slowly released as the substrate degrades *in vivo*. As the cell-substrate composite is vascularized and the structure degrades, the cells will differentiate according to their inherent characteristics.

[0037] The reconstituted cartilage tissue of the present invention may be prepared from chondrocytes isolated from articular cartilage from animals, preferably humans. A particularly useful system may be prepared from chondrocytes isolated from human articular cartilage, for example from the knee joint. The chondrocytes may be isolated by sequential enzyme digestion techniques, such as those described in Kandel et al., Biochem. Biophys. Acta. 1035:130, 1990. For example, the cartilage may be treated with 0.5% protease followed by 0.04% bacterial collagenase.

[0038] The living cells contact an exterior portion of the substrate. Usually, the cells will be cultured *in vitro* on the substrate. The support material and living cells may then be placed in contact with living tissue to function as a tissue repair device.

[0039] The chondrocytes seeded on the coated substrate, may be grown in suitable culture conditions. Broadly, *in vitro* cell cultivation involves harvesting the desired cells from the host organism, placing them on the substrate, and providing an appropriate nutrient medium and temperature and atmospheric conditions to allow the cells to grow and divide. In general, the nutrient medium contains amino acids, vitamins, salts, glucose and antibiotics. The support material impregnated with cells is placed in a suitable container in communication with the appropriate nutrient medium and maintained in an incubator until the desired number of cell divisions have occurred.

[0040] Examples of suitable culture media are known in the art, such as Ham's F12 medium. The culture medium may contain serum, for example fetal bovine serum in a concentration range of about 2-15% and may further contain growth factors and ascorbic acid. The cells may be cultured at 37° C. in a humidified atmosphere supplemented with CO₂, for at least 2 weeks.

[0041] The cells, after harvest from an organism, either as an intact tissue structure or as a suspension formed, e.g., by centrifugation or ultrasonic tissue disruption, are placed in contact with a support material. Exemplary techniques for forming cell suspensions are described in U.S. Pat. Nos. 4,418,691, 4,458,678, 4,505,266, and 5,041,138.

[0042] Following formation of a suspension, the support material must be impregnated with the cells such that the cells are located on the surfaces of the various individual fibers. This impregnation may be accomplished in a variety of ways depending upon the shape and thickness of the support material. For example, the support material of the present invention may be im-

pregnated by immersion in a cell suspension, spraying with a cell suspension, injection with a cell suspension via hypodermic needle, centrifugation of the support material with a cell suspension or any other technique in the art for impregnating a material with a suspension. Usually, the impregnation step is accomplished so that the cells are placed not merely on the surface of a prosthetic implant, but are located throughout the material, adhering to the surface of the support material. The ability to situate cells on the substrate permits a higher density of cells within a given implant. As a result, faster rates of tissue formation throughout the device as well as enhanced tissue ingrowth rates and vascularization following implantation of a seeded device into the host organism are achieved.

[0043] It is within the scope of this invention to coat or impregnate the support material with, or otherwise apply thereto, one or more materials which enhance its functionality, e.g., surgically useful substances, such as those which accelerate or beneficially modify the healing process when the support material is applied to a graft site. Additionally, components of the extracellular matrix, e.g., polysaccharides and proteins such as collagen, can be coated onto the prosthetic support material of the invention to enhance cell growth and division.

[0044] As indicated, the reconstituted cartilage tissue of the present invention also can be used as a model system for *in vitro* studies of cartilage structure, function and development. In particular, the reconstituted cartilage of the present invention may be used in the testing of pharmaceutical preparations useful in the treatment of diseases of the joint, for example osteoarthritis, inflammatory arthropathies, septic arthritis and crystalline arthropathies.

[0045] The reconstituted cartilage tissue of the invention is principally used as an implant for use in the joints of patients to replace or repair damaged or deficient cartilage.

Claims

1. A biological material comprising cartilage tissue and a substrate, the material being obtainable by reconstituting cartilage tissue on a substrate *in vitro* from isolated chondrocytes wherein the substrate is a continuous, flexible sheet of demineralized natural bone, having a thickness less than about 1.5 mm.
2. A method for producing a biological material comprising reconstituted cartilage tissue, wherein the method comprises isolating chondrocytes from cartilage tissue, contacting a substrate comprised of a continuous, flexible sheet of demineralized natural bone, having a thickness less than about 1.5 mm, with said chondrocytes, and culturing the chondrocytes on the substrate in growth media to produce

a biological material characterized by a continuous layer of cartilage tissue.

3. The method of claim 2 wherein the chondrocytes are isolated from human articular cartilage tissue.
4. The method of claim 3, wherein the chondrocytes are isolated by sequential enzyme digestion techniques.
5. A method for making a cartilaginous structure comprising providing a substrate comprised of a continuous, flexible sheet of demineralized natural bone, having a thickness less than about 1.5 mm, in a nutrient environment and attaching cartilage cells to the substrate *in vitro* to form a cartilaginous structure suitable for implantation into a patient to replace defective or missing cartilage.
6. The method of claim 5 further comprising proliferating the cells on the substrate *in vitro* in a nutrient media, thereby to produce a structure comprising the expanded cells on the substrate.
7. A biological material as obtainable by the method of any one of claims 2 to 4.
8. A cartilaginous structure as obtainable by the method of claim 5 or claim 6.
9. A biological material of claim 1 or claim 7, or a cartilaginous structure of claim 8 for implantation in a human or animal body.
10. Use of a biological material of claim 1 or claim 7, or of a cartilaginous structure of claim 8 in the manufacture of an implant for implantation in a human or animal body.
11. Use according to claim 10 wherein the implant is for implantation without prior *in vitro* proliferation, on the substrate, of cells attached to the substrate.
12. Use according to claim 10 wherein the implant is for the repair of cartilage damaged by inflammation, trauma or ageing, or for the repair of cartilage which is congenitally defective.

Patentansprüche

1. Biologisches Material, das Knorpelgewebe und ein Substrat umfasst, wobei das Material durch Wiederaufbau von Knorpelgewebe von isolierten Chondrozyten auf einem Substrat *in vitro* erhältlich ist, wobei das Substrat eine kontinuierliche, flexible Lage aus entmineralisiertem natürlichem Knochen mit einer Dicke von weniger als etwa 1,5 mm ist.

2. Verfahren zur Herstellung von biologischem Material, das wiederaufgebautes Knorpelgewebe umfasst, worin das Verfahren folgende Schritte umfasst: das Isolieren von Chondrozyten aus Knorpelgewebe, das Kontaktieren eines Substrats, das aus einer kontinuierlichen flexiblen Lage aus entmineralisiertem natürlichem Knochen besteht und eine Dicke von weniger als etwa 1,5 mm aufweist, mit den Chondrozyten, sowie das Kultivieren der Chondrozyten auf dem Substrat in Wachstumsmedium, um ein biologisches Material zu erzeugen, das durch eine kontinuierliche Schicht Knorpelgewebe gekennzeichnet ist.
3. Verfahren nach Anspruch 2, worin die Chondrozyten aus menschlichem Gelenkknorpelgewebe isoliert werden.
4. Verfahren nach Anspruch 3, worin die Chondrozyten durch aufeinander folgende Enzymverdautechiken isoliert werden.
5. Verfahren zur Herstellung einer knorpelartigen Struktur, welches das Bereitstellen eines Substrats, das aus einer kontinuierlichen flexiblen Lage aus entmineralisiertem natürlichem Knochen besteht und eine Dicke von weniger als etwa 1,5 mm aufweist, in einer Nährstoffumgebung und das Binden von Knorpelzellen an das Substrat *in vitro* umfasst, um eine knorpelartige Struktur zu bilden, die sich zum Implantieren in einen Patienten als Ersatz für geschädigten oder fehlenden Knorpel eignet.
6. Verfahren nach Anspruch 5, das weiters das Vermehren der Zellen auf dem Substrat *in vitro* in einem Nährstoffmedium umfasst, um dadurch eine Struktur zu erzeugen, die die expandierten Zellen auf dem Substrat umfasst.
7. Biologisches Material, das nach einem Verfahren nach einem der Ansprüche 2 bis 4 erhältlich ist.
8. Knorpelartige Struktur, die nach einem Verfahren nach Anspruch 5 oder 6 erhältlich ist.
9. Biologisches Material nach Anspruch 1 oder 7 oder knorpelartige Struktur nach Anspruch 8 zum Implantieren in einen Menschen- oder Tierkörper.
10. Verwendung eines biologischen Materials nach Anspruch 1 oder 7 oder einer knorpelartigen Struktur nach Anspruch 8 bei der Herstellung eines Implantats zum Implantieren in einen Menschen- oder Tierkörper.
11. Verwendung nach Anspruch 10, worin das Implantat dazu bestimmt ist, an das Substrat gebundene Zellen ohne vorherige Vermehrung *in vitro* auf dem

Substrat zu implantieren.

12. Verwendung nach Anspruch 10, worin das Implantat zur Wiederherstellung von durch Entzündung, Trauma oder Alterung geschädigten Knorpel oder zur Wiederherstellung von kongenital geschädigtem Knorpel bestimmt ist.

10 Revendications

1. Matériau biologique comprenant un tissu de cartilage et un substrat, le matériau pouvant être obtenu par reconstitution d'un tissu de cartilage sur un substrat *in vitro* à partir de chondrocytes isolés, où le substrat est une feuille flexible continue d'os naturel déminéralisé ayant une épaisseur inférieure à environ 1,5 mm.
2. Méthode de production d'un matériau biologique comprenant du tissu de cartilage reconstitué, où la méthode comprend l'isolement de chondrocytes du tissu de cartilage, la mise en contact d'un substrat composé d'une feuille flexible continue d'os naturel déminéralisé ayant une épaisseur inférieure à environ 1,5 mm avec lesdits chondrocytes et la culture de chondrocytes sur le substrat dans des milieux de croissance pour produire un matériau biologique caractérisé par une couche continue d'un tissu de cartilage.
3. Méthode de la revendication 2, où les chondrocytes sont isolés d'un tissu de cartilage articulaire humain.
4. Méthode de la revendication 3, où les chondrocytes sont isolés par technique de digestion d'enzyme séquentielle.
5. Méthode de production d'une structure cartilagineuse consistant à produire un substrat composé d'une feuille flexible continue d'os naturel déminéralisé ayant une épaisseur inférieure à environ 1,5 mm dans un environnement nutritif et attacher de cellules de cartilage au substrat *in vitro* pour former une structure cartilagineuse appropriée pour une implantation chez un patient pour remplacer un cartilage défectueux ou manquant.
6. Méthode de la revendication 5 comprenant de plus la prolifération des cellules sur le substrat *in vitro* dans un milieu nutritif pour ainsi produire une structure comprenant les cellules étendues sur le substrat.
7. Matériau biologique tel qu'il peut être obtenu par la méthode de l'une quelconque des revendications 2 à 4.

8. Structure cartilagineuse telle qu'elle peut être obtenue par la méthode de la revendication 5 ou la revendication 6.
9. Matériau biologique de la revendication 1 ou la revendication 7, ou une structure cartilagineuse de la revendication 8 pour implantation dans un corps humain ou animal. 5
10. Utilisation d'un matériau biologique de la revendication 1 ou la revendication 7, ou d'une structure cartilagineuse de la revendication 8 dans la fabrication d'un implant pour implantation dans un corps humain ou animal. 10
11. Utilisation selon la revendication 10, où l'implant est pour implantation sans prolifération antérieure *in vitro* sur le substrat de cellules attachées au substrat. 15
12. Utilisation selon la revendication 10, où l'implant est pour la réparation d'un cartilage endommagé par une inflammation, un traumatisme ou un vieillissement, ou pour la réparation d'un cartilage qui est congénitalement défectueux. 20 25

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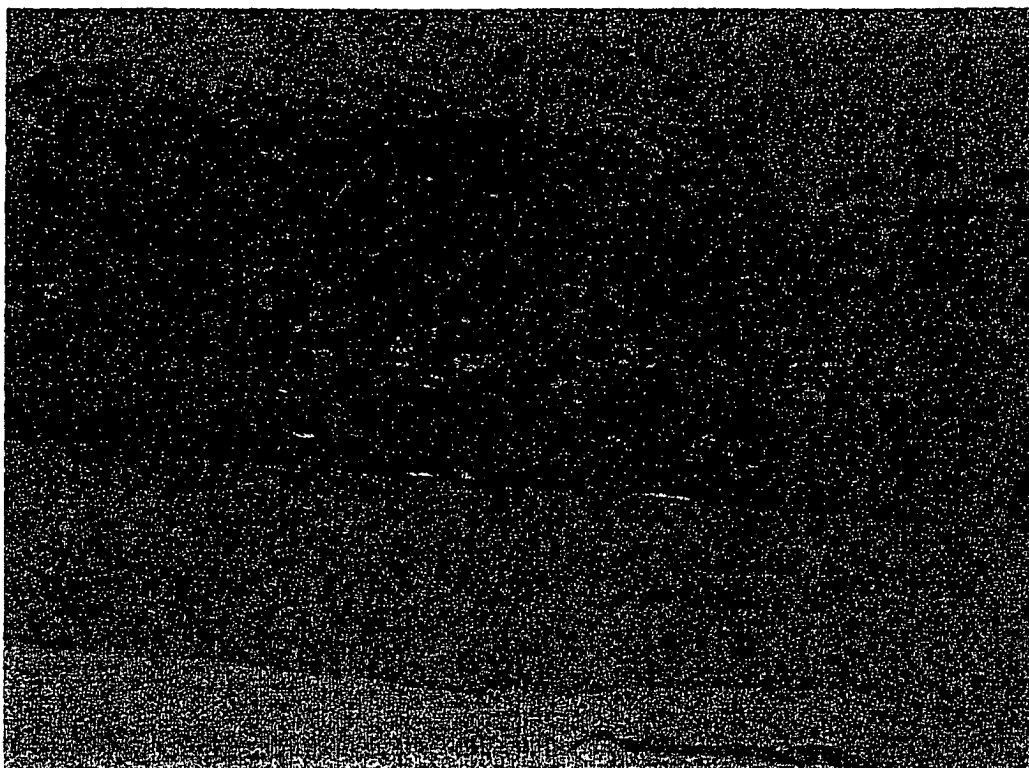


Figure 1

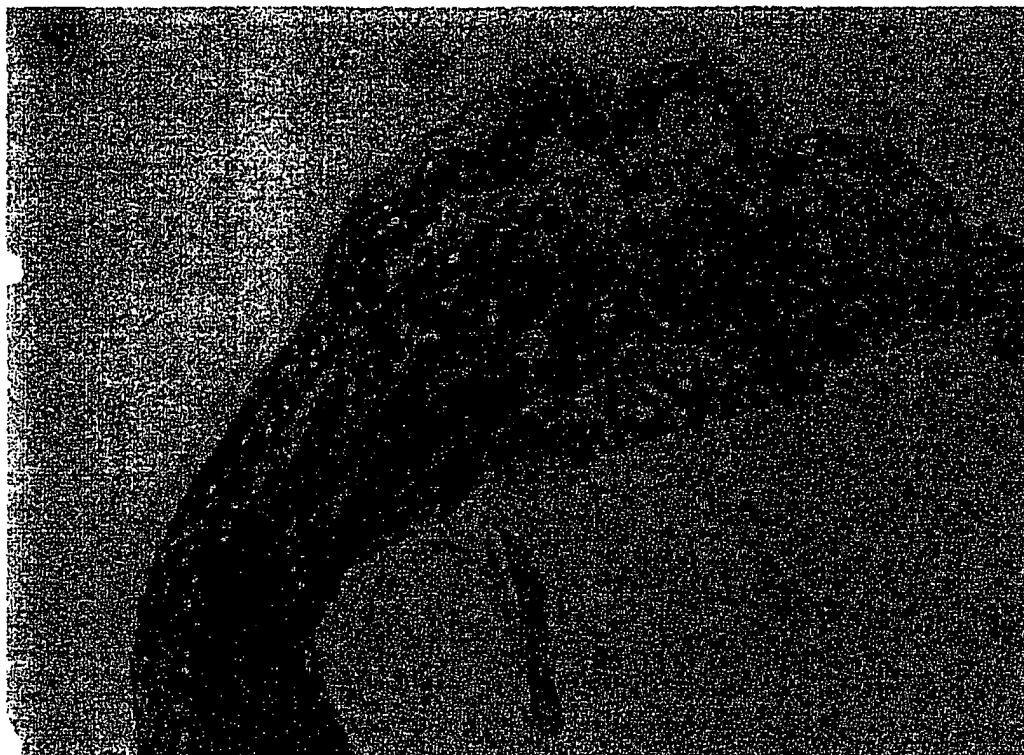


Figure 2